Exhibit 1

IL-12 Is a Central Mediator of Acute Graft-Versus-Host Disease in Mice[1]

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Distinct forms of graft-vs-host disease (GVHD) occur in (C57Bl/6 × DBA/2)F_1 (BDF_1) mice inoculated with either C57Bl/6 or DBA/2 parental spleen cells, and it has been suggested that this reflects differential activation of $CD4^+$ Th cell subsets. Transfer of B6 cells produces an acute GVHD, during which an early period of lymphoid hyperplasia precedes immunosuppression, weight loss, and mortality, and a Th1 pattern of cytokines is produced. Conversely, transfer of DBA/2 cells induces a chronic GVHD, in which no weight loss or mortality is observed, but an autoimmune, SLE-like GVHD develops in association with a Th2 pattern of cytokines. Recent work indicates that IL-12 plays a central role in the polarization of Th cell-dependent responses, and here we have examined its role in polarizing GVHD, by administering or depleting IL-12 during the afferent phase of both the acute and chronic forms of GVHD in BDF_1 mice. In vivo neutralization of endogenous IL-12 ameliorated acute GVHD, in association with reduced splenic NK cell activity, IFN-γ production, immunosuppression, weight loss, and mortality. Conversely, administration of exogenous murine rIL-12 exacerbates this disease and converts the chronic GVHD into a lethal acute GVHD-like syndrome. These results indicate that IL-12 plays an important role in the development of acute, but not chronic, GVHD and suggest that differential production of IL-12 early in the disease may underlie these distinct outcomes of the GVHD in BDF_1 mice injected with different parental cells. *The Journal of Immunology,* **1996, 157: 689–699.**

The distinct forms of GVHD[4] that occur in BDF_1 mice given C57Bl/6 (B6) or DBA/2 parental lymphocytes provide useful experimental models to examine the pathogenesis of GVHD and other immunologically mediated diseases. B6 donor cells produce an acute, immunosuppressive GVHD, in which early lymphoid hyperplasia and heightened NK cell activity are followed by the generation of anti-host CTL, depression of other T cell function, weight loss, and, ultimately, death (1). Conversely, F_1 mice inoculated with DBA/2 parental cells develop a chronic immunostimulatory GVHD associated with B cell hyperplasia, autoantibody production, and immune complex glomerulonephritis (1–3). In addition, the anti-host CTL activity and immunosuppression found in the acute disease are not evident in F_1 recipients of DBA/2 cells (2, 4).

Previous studies of these models suggested that the development of acute GVHD is associated with the production of IL-2 (5) and IFN-γ (6, 7). In contrast, the chronic form of disease is characterized by the production of IL-4, IL-5, and IL-10 (6, 8) and is prevented by in vivo neutralization of IL-4 (9). Together, these findings suggest that the development of the distinct forms of GVHD in BDF_1 hosts may reflect differential production of cytokines by distinct $CD4^+$ Th subsets. However, the underlying cause of this polarization remains unclear.

In other models of T cell-mediated immunity, the differentiation of Th1 and Th2 cells from naive $CD4^+$ T cells appears to be influenced by the early production of cytokines by cells of the innate immune system (10, 11). Thus, the presence of IL-4 during the initial period of immune stimulation promotes the development of a Th2-type response (12–14), while IL-12 and IFN-γ, produced by macrophages and NK cells, respectively, initiate Th1 cell differentiation (15–17).

Previous studies reported that the autoimmune consequences of chronic GVHD in BDF_1 mice can be prevented by the administration of rmIL-12 (18), suggesting that this cytokine may have an important influence on the nature of GVHD that develops in these animals. Here we have examined further the role of IL-12 in polarizing GVHD by investigating the effects of administering or depleting IL-12 on both the acute and chronic forms of GVHD in BDF_1 mice.

Materials and Methods

Mice

Female C57Bl/6 ($H\text{-}2^b$), DBA/2 ($H\text{-}2^d$), and (C57Bl/6 × DBA/2)F_1 (BDF_1) ($H\text{-}2^{bxd}$) mice were obtained from Harlan Olac (Bicester, Oxon, U.K.). Mice were specified pathogen free and were maintained under standard animal housing conditions until use at 6 to 8 wk of age.

Induction of GVHR

The GVHR was induced by i.v. injection of 10^8 viable C57Bl/6 or DBA/2 spleen cells into BDF_1 recipients. Control mice received RPMI only. The intensity of the systemic GVHR was assessed in BDF_1 mice by measurements of splenomegaly and body weight. Splenomegaly was expressed as the spleen index, with an index of >1.3 considered indicative of significant GVHR (19).

Treatment of mice with anti-IL-12 antibody

BDF_1 mice received 0.5 mg of goat IgG anti-mouse IL-12 Ab (20) (kindly provided by Dr. M. K. Gately, Hoffmann-La Roche, Nutley, NJ) i.p. 1 day before the induction of GVHR and then subsequently on days 2, 5, and 8

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[4] Abbreviations used in this paper: GVHD, graft-vs-host disease; rmIL-12, recombinant murine interleukin-12; GVHR, graft-vs-host reaction.

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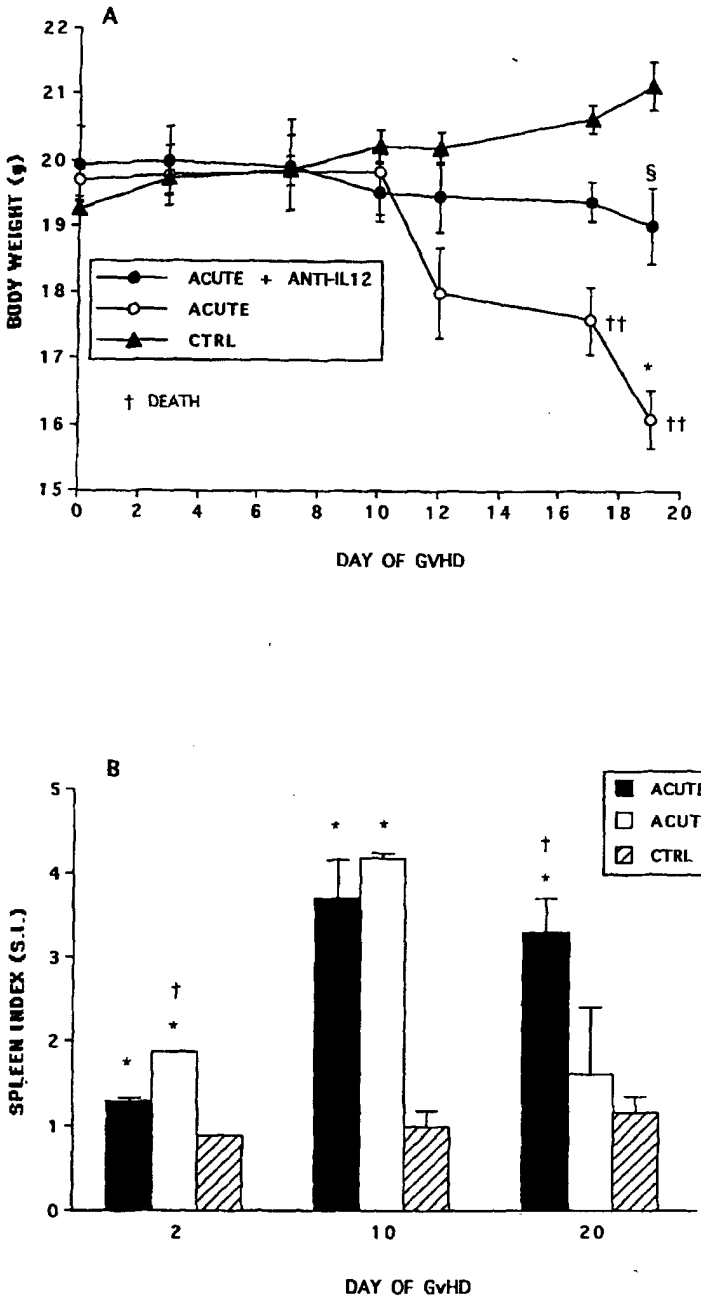

FIGURE 1. Effect of IL-12 depletion on systemic indexes of acute GVHD. *A*, Weight loss and mortality. Results are shown as the mean body weight ± 1 SEM for six mice per group. * Indicates $p < 0.05$ vs controls; § indicates $p < 0.05$ vs untreated acute GVHD; † indicates death. *B*, Splenomegaly in mice with GVHD. Results are shown as the mean spleen indexes ± 1 SD for three mice per group. * Indicates $p < 0.001$ vs controls; † indicates $p < 0.001$ vs untreated acute GVHD.

of the disease. Control animals received 0.5 mg of normal goat IgG (Sigma Chemical Co., St. Louis, MO).

Treatment of mice with IL-12

BDF_1 mice were injected i.p. with 100 ng rmIL-12 (21) (from Dr. M. K. Gately) diluted in PBS containing 1% syngeneic mouse serum. rmIL-12 was administered daily from days −1 to 4 and then from days 7 to 11 of GVHR.

Preparation of spleen cell suspensions and assessment of lymphocyte proliferation in vitro

Single cell suspensions were prepared in RPMI 1640 (Life Technologies, Grand Island, NY) by rubbing spleens from three GVHR or control mice through a stainless steel mesh and passing the resulting suspension through Nitex mesh (Cadisch & Sons, London, U.K.). After three washes in medium, the cells were resuspended at a final concentration of 10^6 cells/ml and cultured in 200-μl aliquots in flat-bottom, 96-well tissue culture plates (Costar, Nucleopore, High Wycombe, U.K.) in medium containing 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 25 mM HEPES, and 0.05 M 2-ME (all from Life Technologies), either alone or with 10 μg/ml Con A (Sigma Chemical Co.). Proliferation was assessed by the addition of 1 μCi/well [^{3}H]thymidine for the last 24 h of culture. Cell-bound DNA was harvested on filter mats, and [^{3}H]thymidine incorporation was measured on a Betaplate counter (Wallac, Turku, Finland).

Induction and measurement of cytokine production in vitro

Single spleen cell suspensions were resuspended at a final concentration of 4×10^6 cells/ml and cultured in 1-ml aliquots in 24-well tissue culture plates (Costar) either in medium alone or in medium with 10 μg/ml Con A. After harvesting, supernatants were centrifuged at 13,000 rpm to remove cellular debris and stored at −20°C until assayed. Cytokine production was quantified using sandwich ELISA techniques, as described in detail previously (22). Cytokine concentrations were calculated with reference to standard curves constructed using recombinant cytokines.

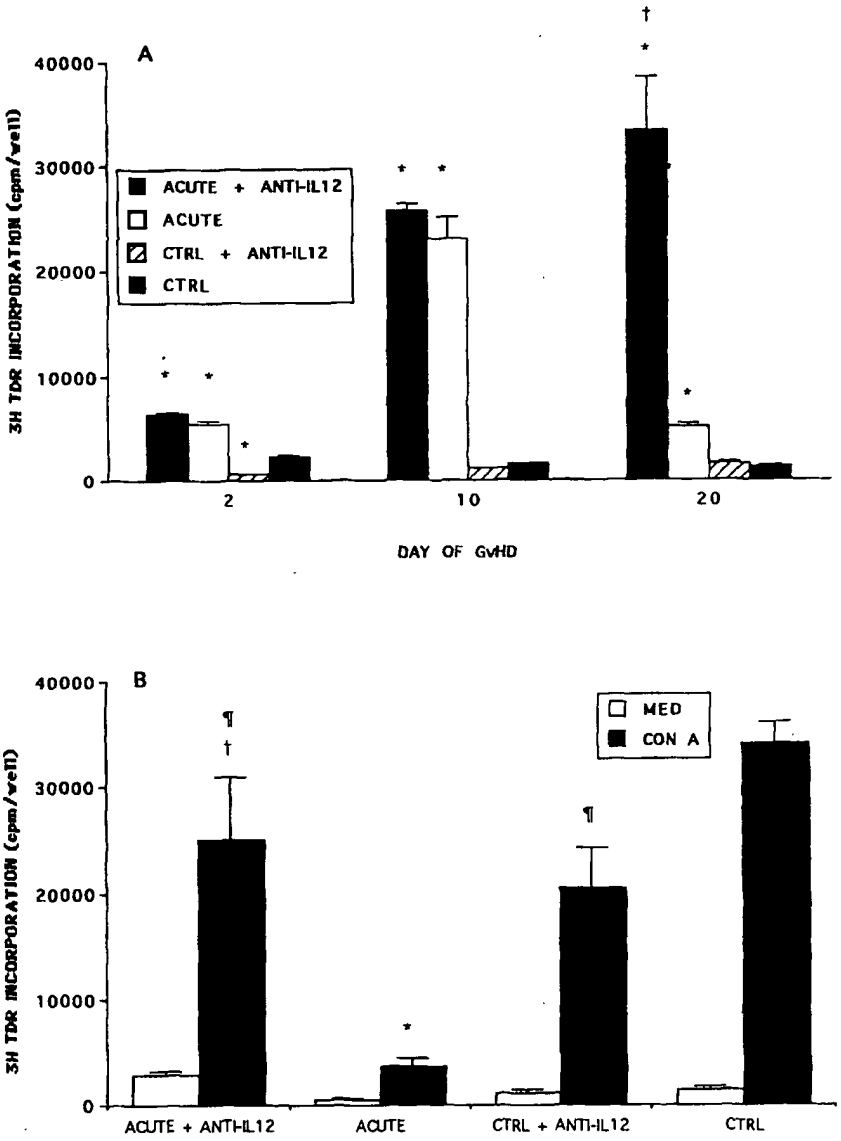

FIGURE 2. Effect of anti-IL-12 treatment on lymphocyte function in acute GVHD. *A*, Spontaneous ex vivo proliferation. Results show the proliferative capacity of splenocytes during acute GVHD, expressed as the mean uptake of [^{3}H]TdR ± 1 SD after 4 h in quadruplicate cultures using spleen cells pooled from three mice per group. *B*, Con A-induced proliferation. Results show the proliferative capacity of splenocytes in response to mitogenic stimulation on day 20 of acute GVHD, expressed as the mean uptake of [^{3}H]TdR ± 1 SD after 48 h in quadruplicate cultures using spleen cells pooled from three or four mice per group. Med indicates the background splenocyte proliferation for each group. * Indicates $p < 0.001$ vs controls; † indicates $p < 0.001$ vs untreated acute GVHD; ¶ indicates $p < 0.01$ vs controls.

Measurement of specific and nonspecific cell-mediated cytotoxicity

Specific anti-host CTL activity was measured in the spleens of GVHR mice using P815 (H-2^d) or EL-4 (H-2^b) target cells, while NK cell activity was measured using YAC-1 cells as targets. As described in detail previously (23), target cells were labeled for 1 h at 37°C with 50 μCi ^{51}Cr/2 × 10^7 cells, and 100-μl aliquots were then added to the wells of V-bottom microtiter plates (Titertek, Flow Laboratories, Rickmansworth, U.K.). Spleen cells from three GVHR or control mice were pooled in RPMI 1640/5% newborn calf serum, and 100-μl aliquots were added to the microtiter plates to achieve E:T ratios of 100:1, 50:1, 25:1, and 12.5:1. The plates were incubated at 37°C for 4 h, and the percent cytotoxicity was calculated by the following formula: % cytotoxicity = (experimental release − spontaneous release)/(maximum release − spontaneous release).

Statistical analysis

Results are represented as the mean ± 1 SD where indicated and were analyzed using Student's *t* test.

Results

Role of endogenous IL-12 in acute and chronic GVHD

Acute GVHD. 1) Systemic indexes of GVHR: BDF$_1$ mice injected with B6 parental cells developed an acute GVHD with progressive weight loss from day 12 until the end of the experiment on day 20 (Fig. 1*A*). Additionally, two of these mice died on day 17, and two additional animals died on day 19. In contrast, BDF$_1$ mice given B6 spleen cells and treated with anti-IL-12 Ab showed no significant weight loss, and none of these mice died.

In parallel, mice with acute GVHD developed significant splenomegaly by day 2, and this peaked on day 10 before returning toward control values. Treatment with anti-IL-12 Ab significantly reduced the degree of splenomegaly on day 2, but by day 10 and thereafter, these mice had similar or even greater splenomegaly than untreated mice with acute GVHD (Fig. 1*B*).

2) Lymphocyte function in acute GVHD: The splenomegaly in untreated mice with acute GVHD was accompanied by enhanced spontaneous ex vivo proliferation of spleen cells compared with that of control cells. This was apparent by day 2, peaked on day 10, and was markedly reduced again by day 20 (Fig. 2*A*). Treatment with anti-IL-12 Ab did not alter the enhanced spontaneous proliferation seen on days 2 and 10, but on day 20, the proliferative capacity of anti-IL-12-treated acute GVHD spleen cells remained significantly higher than both untreated acute GVHD and control levels.

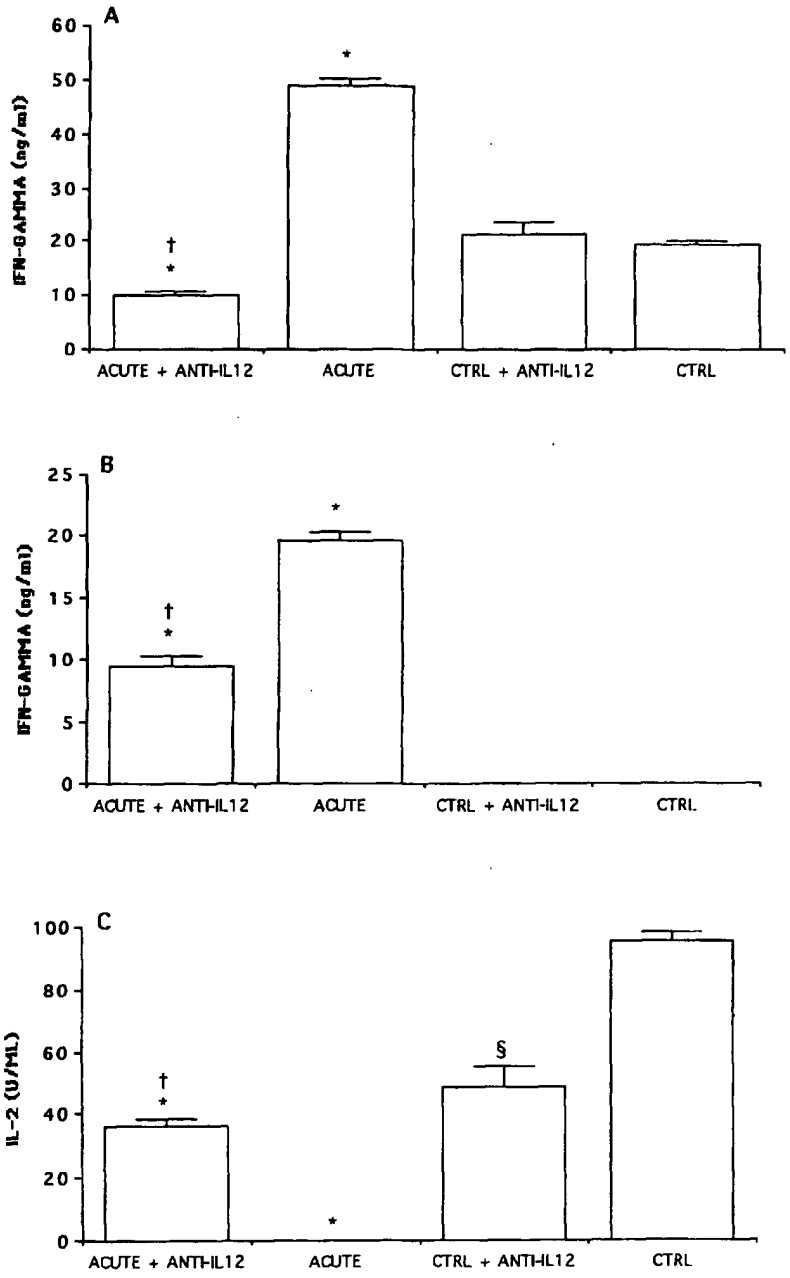

FIGURE 3. Effect of IL-12 depletion on cytokine production during the course of acute GVHD. *A*, Con A-induced IFN-γ production on day 2; *B*, spontaneous IFN-γ production on day 10; *C*, Con A-induced IL-2 production on day 20 of the disease. Results shown are the mean ± 1 SD of triplicate samples from supernatants of cells cultured with Con A for 24 h for IL-2 production and with or without Con A for 48 h for IFN-γ production. * Indicates $p < 0.001$ vs controls; † indicates $p < 0.001$ vs untreated acute GVHD; § indicates $p < 0.005$ vs controls.

Splenocytes from acute GVHD mice had normal proliferative responses to Con A on days 2 and 10 of the disease (data not shown), but by day 20, these responses were significantly lower than those of the controls, consistent with the immunosuppression that characterizes this model of GVHD (1, 24) (Fig. 2*B*). This defect was prevented by treatment with anti-IL-12, as spleen cells from anti-IL-12-treated acute GVHD mice had Con A responses identical with those found in anti-IL-12-treated controls. Anti-IL-12 treatment significantly reduced the spontaneous ex vivo proliferative capacity of control spleen cells on day 2, although by day 10 and thereafter, no reduction was evident (Fig. 2*A*). Anti-IL-12 also caused a significant reduction in the proliferative capacity of control cells in response to Con A stimulation (Fig. 2*B*).

3) Cytokine production: The acute GVHD in untreated mice was characterized by enhanced IFN-γ production compared with that in controls, first evident in response to Con A on day 2 (Fig. 3*A*) and then spontaneously on day 10 (Fig. 3*B*). Treatment of these mice with anti-IL-12 caused a significant reduction in IFN-γ production accompanied by a significant increase in spontaneous IL-10 production on day 2 (acute, 4.6 ± 1.49 ng/ml; acute plus anti-IL-12, 14.83 ± 1.6 ng/ml; $p < 0.001$).

On day 20, the suppressed mitogen responses exhibited by acute GVHD mice were accompanied by a failure of spleen cells to produce the IL-2 made by control cells in response to Con A (Fig. 3*C*). This defect in cytokine production was significantly reduced by treatment with anti-IL-12.

The Ab had no effect on the low level of IFN-γ secretion by control spleen cells in response to Con A, but significantly reduced IL-2 production.

4) Nonspecific and specific cell-mediated cytotoxicity: During the early phase of the disease, on day 2, animals with acute GVHD displayed enhanced levels of splenic NK cell activity compared

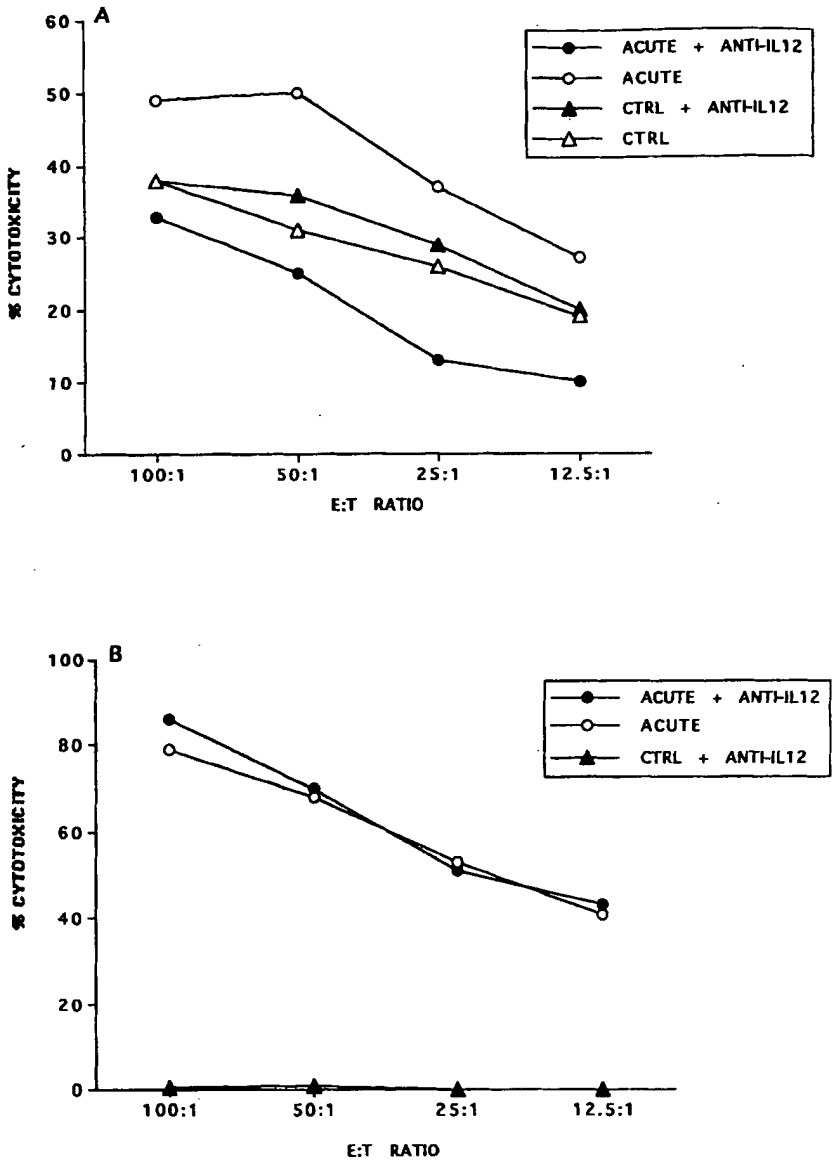

FIGURE 4. Effect of IL-12 depletion on cell-mediated cytotoxicity in mice with acute GVHD. *A*, NK cell activity against YAC-1 targets. *B*, Specific CTL activity against P815 targets, Results shown are the percent cytotoxicity from quadruplicate assays, measured at E:T ratios from 100:1 to 12.5:1, using spleen cells pooled from three mice per group. Similar results were obtained in two replicate experiments.

with those in controls. Treatment with anti-IL-12 Ab reduced this activity to below control levels, but had no effect on NK cell activity in control mice (Fig. 4*A*).

Despite this effect on NK cell activation and its ability to ameliorate the associated weight loss and mortality, anti-IL-12 did not prevent the generation of the anti-host CTL that accompany the pathologic features of acute GVHD (Fig. 4*B*).

Chronic GVHD. As described in detail below, BDF_1 mice given DBA/2 parental cells did not exhibit the weight loss or early mortality observed in the acute disease. In parallel, mice with chronic GVHD exhibited less pronounced lymphoid hyperplasia than mice with acute GVHD, and anti-host CTL activity was never detected, although some enhancement of NK cell activity was observed (data not shown). Treatment with anti-IL-12 had no effect on any of these parameters and did not affect the cytokine profile associated with the chronic disease, which is characterized by low levels of IFN-γ and enhanced levels of IL-10 compared with control values (data not shown).

Together, these results indicate that endogenous IL-12 appears to play an integral role in the initiation of acute, but not chronic, GVHD. To explore further the influence of IL-12, we examined the effect of administering exogenous rmIL-12 to both acute and chronic GVHD mice.

Modulation of GVHD by administration of exogenous IL-12

Systemic features of chronic and acute GVHD. As before, BDF_1 mice with chronic GVHD showed no evidence of weight loss or mortality for the duration of this study (Fig. 5*A*). However, similar mice injected with 100 ng of rmIL-12 for 14 days developed an acute GVHD-like syndrome, with progressive weight loss from day 12 and 100% mortality by day 15. rmIL-12 also exacerbated the systemic consequences of acute GVHD, with weight loss and mortality appearing in the IL-12-treated group, but not in the untreated mice with acute GVHD by the time the experiment was terminated (Fig. 5*A*). rmIL-12 did not provoke weight loss or mortality in control animals.

In parallel, chronic GVHD mice treated with IL-12 had significantly greater splenomegaly than the untreated chronic GVHD group by day 10 (Fig. 5*B*). IL-12 also enhanced the splenomegaly in mice with acute GVHD and provoked significant splenomegaly

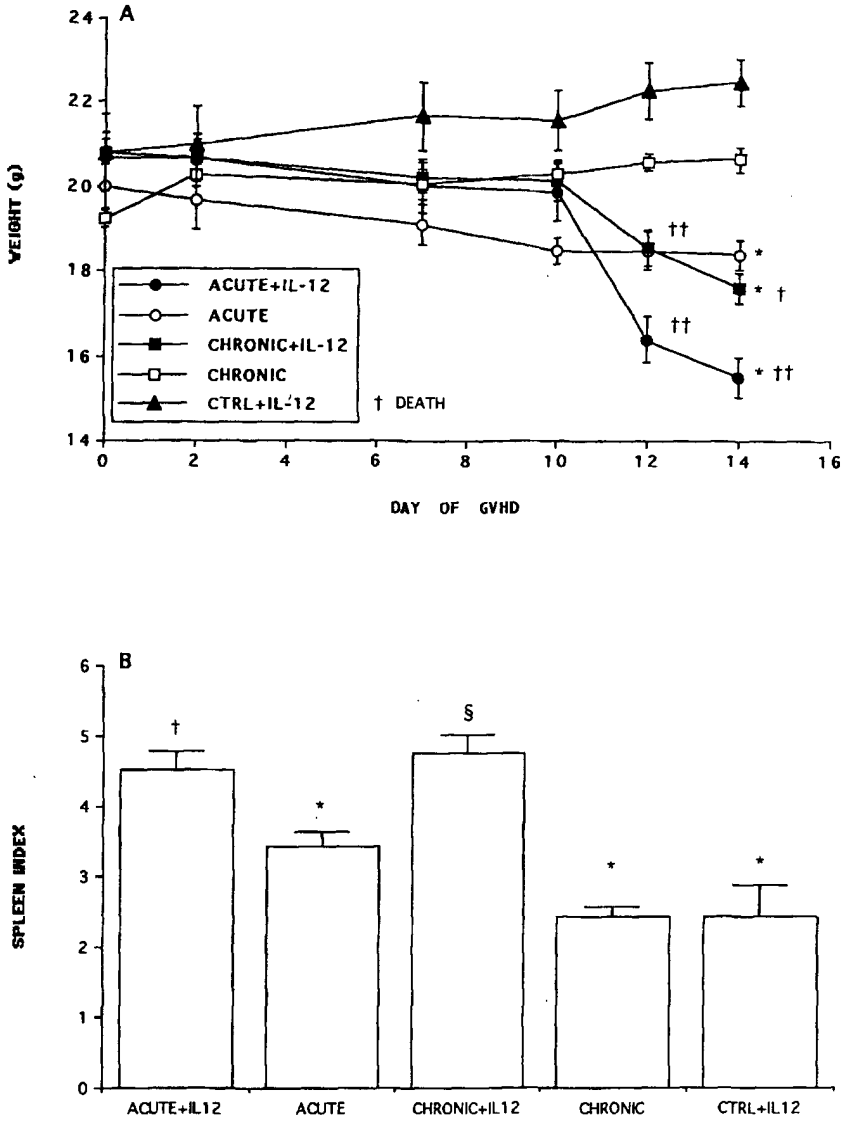

FIGURE 5. Effect of exogenous rmIL-12 administration on systemic indexes of acute and chronic GVHD. *A*, Weight loss and mortality. Results shown are the mean body weight ± 1 SEM for six mice per group. * indicates $p < 0.05$ vs controls. *B*, Splenomegaly. Results shown are mean spleen indexes ± 1 SD for three mice per group. * Indicates $p < 0.005$ vs controls; † indicates $p < 0.005$ vs untreated acute GVHD; § indicates $p < 0.001$ vs untreated chronic GVHD.

in control animals on both days 1 and 10 (Fig. 5*B*). This IL-12-induced splenomegaly in control animals was consistent with the increased hemopoiesis observed in previous studies of IL-12-treated normal mice (21).

Lymphocyte function. Untreated chronic GVHD spleen cells showed significantly enhanced background proliferation compared with control cells on both days 1 (data not shown) and 10 of GVHD, although this was less marked than that found in acute GVHD (Fig. 6*A*). Treatment with rmIL-12 had no effect on the proliferation of chronic GVHD spleen cells on day 1, but by day 10, the rmIL-12-treated chronic GVHD spleen cells showed a marked increase in their spontaneous proliferative capacity, which resembled that observed in mice with acute GVHD. In parallel, IL-12-treated mice with chronic GVHD exhibited significantly suppressed proliferative responses to Con A on day 15, similar to the phenomenon observed in mice with acute GVHD at this time (Fig. 6*B*).

Treatment with rmIL-12 had no effect on the enhanced spontaneous proliferation of acute GVHD spleen cells on day 1, but by day 10, the proliferative capacity of splenocytes from rmIL-12-treated acute GVHD mice was significantly reduced compared with that of untreated mice with acute GVHD (Fig. 6*A*). Although IL-12 slightly increased the low Con A responses of acute GVHD cells on day 15, these remained significantly suppressed compared with those of controls (Fig. 6*B*).

rmIL-12-injected control animals exhibited significantly enhanced spontaneous proliferative responses (Fig. 6*A*), but their Con A responses were normal (Fig. 6*B*).

Cytokine production. Spleen cells from mice with acute GVHD spontaneously produced high levels of IFN-γ from day 1 of the disease. This was not observed in mice with chronic GVHD (Fig. 7*A*), although low levels had appeared by day 10 (Fig. 7*B*). Additionally, the suppression of IL-2 production in response to Con A found in acute GVHD on day 15 was not observed in chronic GVHD (Fig. 7*C*). In contrast, mice with chronic GVHD produced enhanced levels of IL-10 (chronic, 5.44 ± 0.09 ng/ml; control, 1.23 ± 0.74 ng/ml; $p < 0.05$). Administration of IL-12 to mice with chronic GVHD provoked a cytokine profile similar to that associated with acute GVHD, with spontaneous IFN-γ produced by day 1 (Fig. 7*A*), a parallel decrease in IL-10 production (chronic, 5.44 ± 0.09 ng/ml; chronic plus IL-12, 1.70 ± 0.45 ng/ml; $p <$

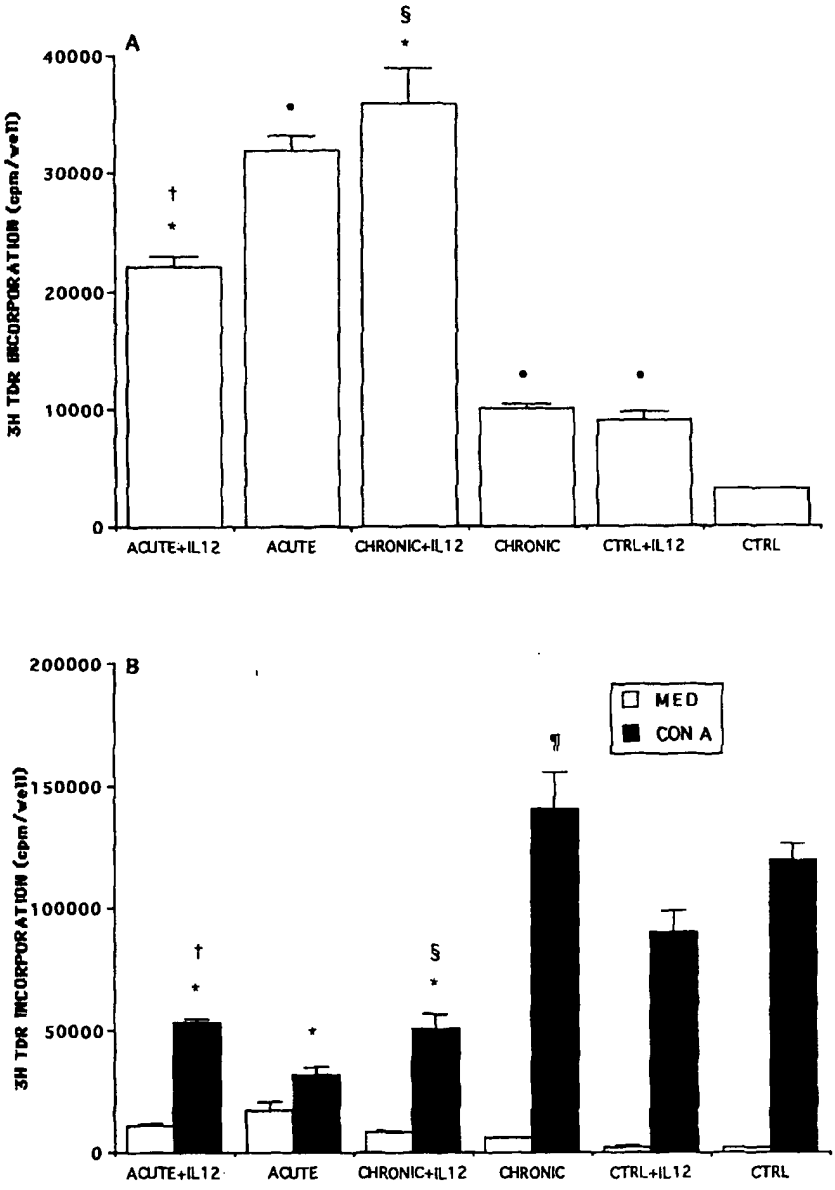

FIGURE 6. Effect of exogenous rmIL-12 administration on lymphocyte function in acute and chronic GVHD. *A*, Spontaneous ex vivo proliferation. Data show the proliferative capacity of splenocytes during acute GVHD, expressed as the mean uptake of [^{3}H]TdR ± 1 SD after 4 h in quadruplicate cultures using spleen cells pooled from three mice per group. *B*, Con A-induced proliferation. Results show the proliferative capacity of splenocytes in response to mitogenic stimulation on day 15 of acute and chronic GVHD, expressed as the mean uptake of [^{3}H]TdR ± 1 SD after 48 h in quadruplicate cultures, using spleen cells pooled from three mice per group. Med indicates the background splenocyte proliferation for each group. * Indicates $p < 0.001$ vs controls; † indicates $p < 0.001$ vs untreated acute GVHD; § indicates $p < 0.001$ vs untreated chronic GVHD; ¶ indicates $p < 0.05$ vs controls.

0.01), and a complete abrogation of IL-2 production in response to Con A by day 15 (Fig. 7*C*).

IL-12 had little effect on the already high levels of IFN-γ or the abrogated levels of IL-2 produced by spleen cells from mice with acute GVHD (Fig. 7, *A* and *C*). It did, however, induce the production of high levels of spontaneous IFN-γ from control spleen cells by day 10 (Fig. 7*B*), which has previously been described (21).

Nonspecific and specific cell-mediated cytotoxicity. NK cell activity was enhanced in the spleens of both chronic and acute GVHD mice on day 1 compared to that in control animals (Fig. 8). Exogenous IL-12 had no effect on any of these levels. Although previous work indicates that giving IL-12 to control mice enhances NK cell activity (21), this increase was observed after two injections of IL-12, while our study used only one injection.

No anti-host CTL activity was found in mice with chronic GVHD, but as before, untreated mice with acute GVHD developed anti-host CTL activity on day 10 of the GVHD (Fig. 9, *A* and *B*). Administration of rmIL-12 induced very high levels of CTL activity in mice with the chronic form of the disease, but suppressed those in acute GVHD, as the peak of CTL activity may have already passed in IL-12-treated mice with more aggressive GVHD.

Overall, therefore, administration of exogenous IL-12 converts the immunologic features of chronic GVHD to a more acute phenotype.

Discussion

The data presented here indicate that in vivo neutralization of endogenous IL-12 inhibits acute GVHD in BDF_1 mice injected with C57Bl/6 spleen cells. Conversely, administration of exogenous rmIL-12 exacerbates this disease and converts the chronic autoimmune GVHD occurring after transfer of DBA/2 parental cells into an acute GVHD-like syndrome. These results suggest that IL-12 plays an important role in the development of acute GVHD and indicate that differential production of IL-12 early in the disease may underlie the distinct forms of the GVHD found in BDF_1 mice given donor cells of different parental origin.

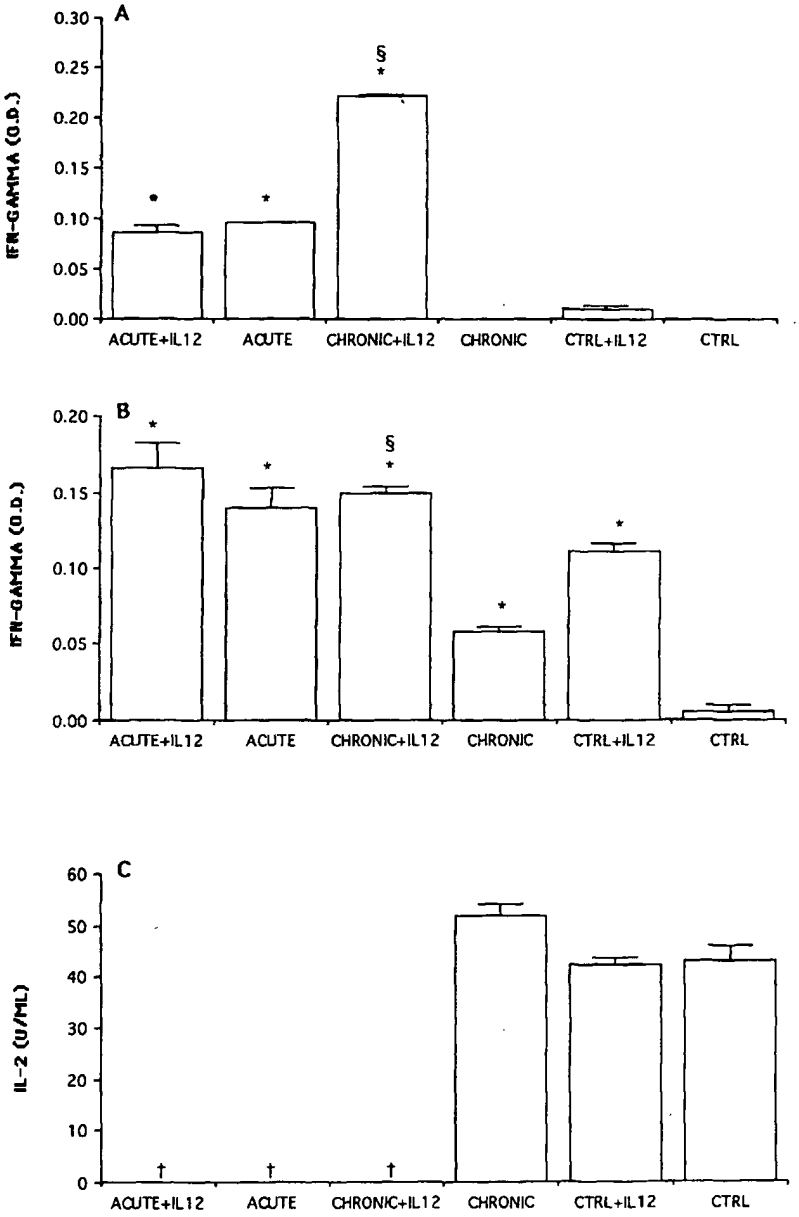

FIGURE 7. Effect of exogenous rmIL-12 administration on cytokine production during the course of acute and chronic GVHD. *A*, Spontaneous IFN-γ production on day 1. *B*, Spontaneous IFN-γ production on day 10. *C*, Con A-induced IL-2 production on day 15 of GVHD. * Indicates $p < 0.001$ vs controls; § indicates $p < 0.001$ vs untreated chronic GVHD; † indicates $p < 0.001$ vs all other groups. All results shown are the mean ± 1 SD of triplicate samples from supernatants of cells cultured with Con A for 24 h for IL-2 production and in medium only for 48 h for IFN-γ production.

The most notable feature of our study was that depletion of IL-12 within the first few days of inducing acute GVHD produced a marked reduction in the immunologic and pathologic consequences of the disease. Although one explanation for this could be that anti-IL-12 Ab impairs the ability of donor T cells to initiate an alloimmune response, we believe this to be unlikely, as spleen cells from anti-IL-12-treated mice exhibited the enhanced ex vivo proliferative capacity usually found in acute GVHD, presumably reflecting the presence of an active allogeneic response. Thus, we propose that the beneficial effects of neutralizing IL-12 are more likely to reflect the down-regulation of pathologic effector mechanisms known to be dependent on IFN-γ, such as immunosuppression (25, 26), intestinal pathology (27), and the recruitment and activation of inflammatory and hemopoietic cells. This explanation is supported by our observations that neutralization of endogenous IL-12 dramatically reduced the high levels of IFN-γ normally present in mice with acute GVHD and that the acute GVHD-like disease we observed when exogenous rmIL-12 was administered to DBA/2 → BDF_1 mice was associated with a striking increase in IFN-γ production. Taken together with the fact that neutralization of IFN-γ itself ameliorates many aspects of acute GVHD (27) (our unpublished observations), this evidence strongly suggests that the destructive pathology observed during acute GVHD is dependent on IL-12-induced IFN-γ production.

This observation also raises important questions about the mechanisms of tissue pathology and mortality in acute GVHD, as previous reports have suggested that mortality in B6 → BDF_1 mice is dependent on activation of $CD8^+$ anti-host CTL (2, 4). However, in our hands, anti-IL-12 prevented mortality in such mice without reducing anti-host CTL activity, which agrees with other studies in which a lack of correlation between anti-host CTL activity and mortality from acute GVHD was observed (28, 29). We believe that anti-IL-12 may prevent mortality by down-regulating IFN-γ production and by promoting preservation of lymphocyte function in these animals, hence reducing their susceptibility to secondary infections.

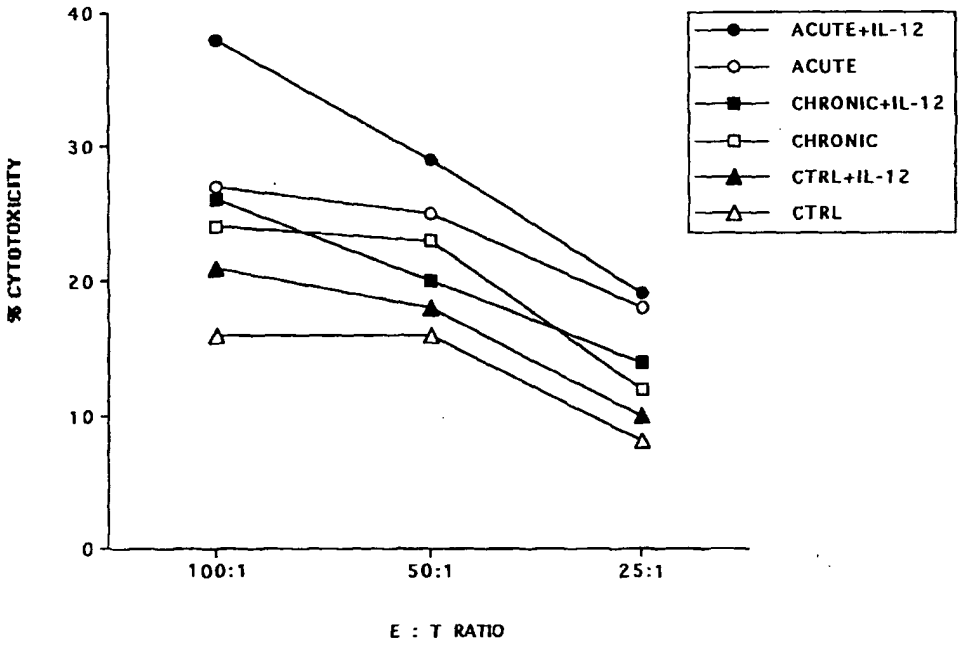

FIGURE 8. Effect of exogenous rmIL-12 administration on NK cell activity in acute and chronic GVHD. Results shown are the percent cytotoxicity from quadruplicate assays against YAC-1 targets, measured at E:T ratios from 100:1 to 25:1, using spleen cells pooled from three mice per group.

The reduced production of IFN-γ could reflect loss of the stimulatory effect of IL-12 on $CD4^+$ Th1 cells, $CD8^+$ T cells, or NK cells (21, 30). Although we have yet to identify the source of IFN-γ in acute GVHD, the possibility that anti-IL-12 may inhibit IFN-γ production by NK cells is supported by the finding that neutralizing IL-12 negated the enhanced NK cell activity normally seen in acute GVHD. Furthermore, NK cells are an important early source of IFN-γ during other models of IFN-γ-dependent immunity (10, 31, 32) and are critical for the development of GVHD (33). Neutralizing IL-12 could also inhibit IFN-γ production by interfering directly with Th1 cell activation, resulting in a direct switch from Th1 to Th2 cell differentiation in vivo (15, 34). This is supported by our finding that the reduced IFN-γ levels we observed after neutralization of IL-12 were paralleled by increased IL-10 production. It will now be important to determine whether anti-IL-12-treated mice go on to develop chronic GVHD, as would be predicted if a polarized Th2 response has been stimulated.

In contrast to the dramatic effects of anti-IL-12 on acute GVHD, the chronic GVHD in DBA/2 → BDF_1 mice was not altered by neutralizing IL-12, indicating that this form of GVHD is not dependent on endogenous IL-12. Indeed, previous studies have demonstrated that the autoimmunity and B cell hyperplasia usually found in mice with chronic GVHD could be prevented by the administration of exogenous rmIL-12 (18). Here we have extended these findings by showing that IL-12 induces an acute GVHD in DBA/2 → mice identical with that found normally in B6 → BDF_1 mice, with a characteristic biphasic pattern of early hyperplasia progressing to activation of CTL, immunosuppression, and death, in association with high levels of IFN-γ.

These results confirm that IL-12 is a central mediator of acute, but not chronic, GVHD. However, the reasons why B6, but not DBA/2, donor cells induce an IL-12-dependent disease in BDF_1 hosts are unclear. It seems unlikely that the polarization of Th responses in this model reflects differential production of IL-12 by APCs, as both forms of GVHD are initiated by recognition of alloantigens presumably expressed on the same host APC. An alternative possibility is that B6 and DBA/2 donor T cells have genetically determined differences in their ability to initiate IFN-γ production in response to APC-derived IL-12. Differences of this nature have been defined recently in MHC congenic BALB/c and B10.D2 mice; although T cells from the two strains are equally capable of initiating Ag-specific Th1 responses, BALB/c T cells become unresponsive to IL-12 and acquire a Th2 phenotype, whereas B10.D2 T cells maintain IL-12 responsiveness and become permanent IFN-γ producers (35). Whether T cells from DBA/2 and C57Bl/6 mice also have intrinsic differences in their ability to sustain IL-12 responsiveness to BDF_1 alloantigen has not been investigated directly. However, it is known that DBA/2 mice preferentially elicit Th2-type responses (36, 37), while mice from the C57 background are biased toward making Th1 responses to a variety of stimuli (35, 36, 38, 39). A disparity at the level of the donor T cells is supported further by previous studies that have attributed the dichotomy between acute and chronic GVHD in BDF_1 mice to differences in the ability of donor $CD8^+$ T cells to engraft and generate allospecific CTL responses (2, 4). For the reasons noted above, we believe that CTL activity itself is not responsible for the lethal consequences of acute GVHD. Nevertheless, other aspects of $CD8^+$ T cell function, such as IFN-γ production, may also differ between B6 and DBA/2 mice and could be a manifestation of inherent differences in the ability of these T cells to respond to IL-12. Thus, it will be important to measure the levels of IL-12 production in the two models of GVHD and to identify the sources of IL-12 and IFN-γ during the disease.

In conclusion, this study provides novel evidence that IL-12 plays a central role in the early stages of acute GVHD and suggests that IL-12 could provide a target for therapy of acute GVHD during the afferent phase of the disease. However, the finding that IL-12 may exacerbate or even provoke immunopathology cautions

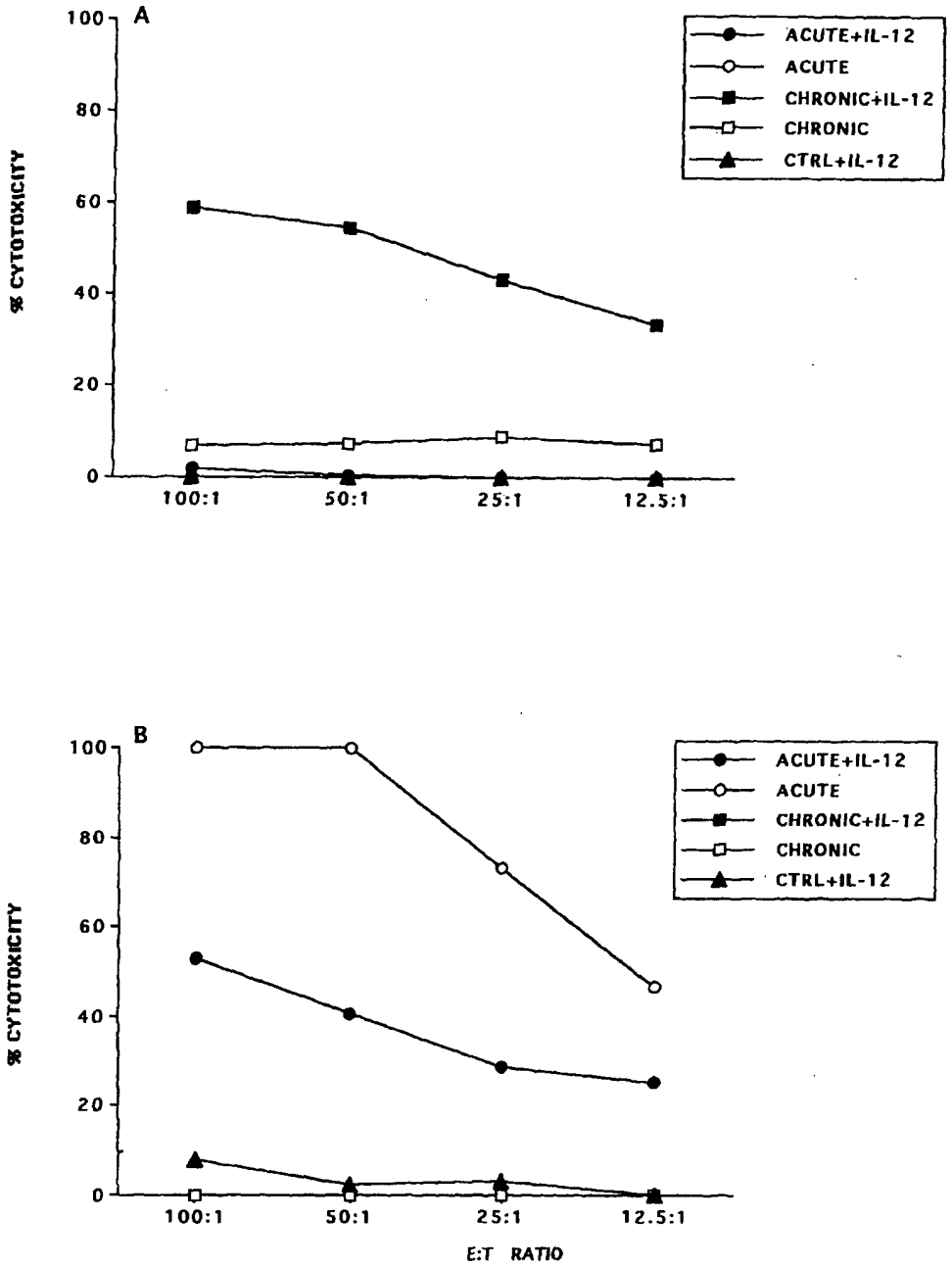

FIGURE 9. Effect of exogenous rmIL-12 administration on specific CTL activity in acute and chronic GVHD. *A*, CTL activity against EL-4 (H-2^b) targets; *B*, CTL activity against P815 (H-2^d) targets. Results shown are the percent cytotoxicity from quadruplicate assays, measured at E:T ratios from 100:1 to 12.5:1, using spleen cells pooled from three mice per group.

the use of this cytokine as a therapeutic agent in patients after bone marrow transplantation.

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References

1. Gleichmann, E., S. T. Pals, A. G. Rolink, T. Radaszkiewicz, and H. Gleichmann. 1984. Graft-versus-host reactions: clues to the etiopathology of a spectrum of immunological diseases. *Immunol. Today 5:324.*
2. Via, C. S., and G. M. Shearer. 1988. T-cell interactions in autoimmunity: insights from a murine model of graft-versus-host disease. *Immunol. Today 9:207.*
3. Gleichmann, E., E. H. van Elven, and J. P. W. van der Veen. 1982. A systemic lupus erythematosus (SLE)-like disease in mice induced by abnormal T-B cell cooperation: preferential formation of autoantibodies characteristic of SLE. *Eur. J. Immunol. 12:152.*
4. Via, C. S., S. O. Sharrow, and G. M. Shearer. 1987. Role of cytotoxic T lymphocytes in the prevention of lupus-like disease occurring in a murine model of graft-versus-host disease. *J. Immunol. 139:1840.*
5. Via, C. S., and F. D. Finkelman. 1993. Critical role of interleukin-2 in the development of acute graft-versus-host disease. *Int. Immunol. 5:565.*
6. Allen, R. D., T. A. Staley, and C. L. Sidman. 1993. Differential cytokine expression in acute and chronic murine graft-versus-host-disease. *Eur. J. Immunol. 23: 333.*
7. Garside, P., S. Reid, M. Steel, and A. McI. Mowat. 1994. Differential cytokine production associated with distinct phases of murine graft-versus-host reaction. *Immunology 82:211.*
8. De Wit, D., M. van Mechelen, C. Zanin, J. Doutrelepont, T. Velu, C. Gerard, D. Abramowicz, J. Scheerlinck, P. De Baetselier, J. Urbain, O. Leo, M. Goldman,

and M. Moser. 1993. Preferential activation of Th2 cells in chronic graft-versus-host reaction. *J. Immunol. 150:361.*

9. Umland, S. P., S. Razac, D. K. Nahrebne, and B. W. Seymour. 1992. Effects of in vivo administration of interferon (IFN)-γ, anti-IFN-γ, or anti-interleukin-4 monoclonal antibodies in chronic autoimmune graft-versus-host disease. *Clin. Immunol. Immunopathol. 63:66.*
10. Romagnani, S. 1992. Induction of T_H1 and T_H2 responses: a key role for the 'natural' immune response? *Immunol. Today 13:379.*
11. Seder, R. A., and W. E. Paul. 1994. Acquisition of lymphokine-producing phenotype by $CD4^+$ T cells. *Annu. Rev. Immunol. 12:635.*
12. LeGros, G., S. Z. Ben-Sasson, R. A. Seder, F. D. Finkelman, and W. E. Paul. 1990. Generation of interleukin 4 (IL-4)-producing cells in vivo and in vitro: IL-2 and IL-4 are required for in vitro generation of IL-4-producing cells. *J. Exp. Med. 172:921.*
13. Seder, R. A., W. E. Paul, M. M. Davis, and B. F. de St. Groth. 1992. The presence of IL-4 during in vitro priming determines the lymphokine-producing potential of $CD4^+$ T cells from T cell receptor transgenic mice. *J. Exp. Med. 176:1091.*
14. Hsieh, C.-S., A. B. Heimberger, J. S. Gold, A. O'Garra, K. M. Murphy. 1992. Differential regulation of T helper phenotype development by interleukins 4 and 10 in an αβ T cell receptor transgenic system. *Proc. Natl. Acad. Sci. USA 89: 6065.*
15. Manetti, R., P. Parronchi, M. G. Giudizi, M. P. Piccinni, E. Maggi, G. Trinchieri, and S. Romagnani. 1993. Natural killer cell stimulatory factor (interleukin-12 [IL-12] induces T helper type 1 (Th1)-specific immune responses and inhibits the development of IL-4-producing Th cells. *J. Exp. Med. 177:1199.*
16. Hsieh, C.-S., S. E. Macatonia, C. S. Tripp, S. F. Wolf, A. O'Garra, and K. M. Murphy. 1993. Development of T_h1 $CD4^+$ T cells through IL-12 produced by *Listeria*-induced macrophages. *Science 260:547.*
17. Schmitt, E., P. Hoehn, C. Huels, S. Goedert, N. Palm, E. Rude, and T. Germann. 1994. T helper type 1 development of naive $CD4^+$ T cells requires the coordinate action of interleukin-12 and interferon-γ and is inhibited by transforming growth factor-β. *Eur. J. Immunol. 24:793.*
18. Via, C. S., V. Rus, M. K. Gately, and F. D. Finkelman. 1994. IL-12 stimulates the development of acute graft-versus-host disease in mice that normally would develop chronic, autoimmune graft-versus-host disease. *J. Immunol. 153:4040.*
19. Simonsen, M. 1962. Graft-versus-host reactions: their natural history and applicability as tools of research. *Prog. Allergy 6:349.*
20. Tripp, C. S., M. K. Gately, J. Hakimi, P. Ling, and E. R. Unanue. 1994. Neutralisation of IL-12 decreases resistance to *Listeria* in SCID and C. B-17 mice: reversal by IFN-γ. *J. Immunol. 152:1883.*
21. Gately, M. K., R. W. Warrier, S. Honasoge, D. M. Carvajal, D. A. Faherty, S. E. Connaughton, T. D. Anderson, U. Sarmiento, B. R. Hubbard, and M. Murphy. 1994. Administration of recombinant IL-12 to normal mice enhances cytolytic lymphocyte activity and induces production of IFN-γ in vivo. *Int. Immunol. 6:157.*
22. Garside, P., M. Steel, E. A. Worthey, A. Satoskar, J. Alexander, H. Bluethmann, F. Y. Liew, and A. McI. Mowat. 1995. T helper 2 cells are subject to high dose oral tolerance and are not essential for its induction. *J. Immunol. 154:5649.*
23. Borland, A., A. McI Mowat, and D. M. V. Parrott. 1983. Augmentation of intestinal and peripheral natural killer cell activity during the graft-versus-host reaction in mice. *Transplantation 36:513.*
24. Shand, F. L. 1976. Analysis of immunosuppression generated by the graft-versus-host reaction. II. Characterisation of the suppressor cell and its mechanism of action. *Immunology 31:943.*
25. Klimpel, G. R., C. R. Annable, M. G. Cleveland, T. R. Jerrells, and J. C. Patterson. 1989. Immunosuppression and lymphoid hypoplasia associated with chronic graft versus host disease is dependent on IFN-γ production. *J. Immunol. 144:84.*
26. Cleveland, M. G., C. R. Annable, and G. R. Klimpel. 1988. In vivo and in vitro production of IFN-β and IFN-γ during graft vs host disease. *J. Immunol. 141: 3349.*
27. Mowat, A. McI. 1989. Antibodies to IFN-γ prevent immunologically mediated intestinal damage in murine graft-versus-host reaction. *Immunology 68:18.*
28. Rolink, A. G., T. Radaszkiewicz, S. T. Pals, W. G. J. van der Meer, and E. Gleichmann. 1982. Allosuppressor and allohelper T cells in acute and chronic graft-vs-host disease. I. Alloreactive suppressor cells rather than killer T cells appear to be the decisive effector cells in lethal graft-vs-host disease. *J. Exp. Med. 155:1501.*
29. Mowat, A. McI., M. V. Felstein, A. Borland, and D. M. V. Parrott. 1988. Experimental studies of immunologically mediated enteropathy: development of cell mediated immunity and intestinal pathology during a graft-versus-host reaction in irradiated mice. *Gut 29:949.*
30. Trinchieri, G. 1995. Interleukin-12: a proinflammatory cytokine with immunoregulatory functions that bridge innate resistance and antigen-specific adaptive immunity. *Annu. Rev. Immunol. 13:251.*
31. Bancroft, G. J., R. D. Schreiber, and E. R. Unanue. 1991. Natural immunity: a T-cell independent pathway of macrophage activation, defined in the *scid* mouse. *Immunol. Rev. 124:5.*
32. Sher, A., I. P. Oswald, S. Hieny, and R. T. Gazzinelli. 1993. *Toxoplasma gondi* induces a T-independent IFN-γ response in natural killer cells that requires both adherent accessory cells and tumor necrosis factor-α. *J. Immunol. 150:3982.*
33. Ghayur, T., T. A. Seemayer, and W. S. Lapp. 1988. Prevention of murine graft-versus-host disease by inducing and eliminating $ASGM1^+$ cells of donor origin. *Transplantation 45:586.*
34. Gazzinelli, R. T., M. Wysocka, S. Hayashi, E. Y. Denkers, S. Hieny, P. Caspar, G. Trinchieri, and A. Sher. 1994. Parasite-induced IL-12 stimulates early IFN-γ synthesis and resistance during acute infection with *Toxoplasma gondii. J. Immunol. 153:2533.*
35. Guler, M. L., J. D. Gorham, C.-S. Hsieh, A. J. Mackey, R. G. Steen, W. F. Dietrich, and K. M. Murphy. 1996. Genetic susceptibility to *Leishmania*: IL-12 responsiveness in T_H1 cell development. *Science 271:984.*
36. Hsieh, C.-S., S. E. Macatonia, A. O'Garra, and K. M. Murphy. 1995. T cell genetic background determines default T helper phenotype development in vitro. *J. Exp. Med. 181:713.*
37. Romani, L., A. Mencacci, E. Cenci, R. Spaccapelo, P. Mosci, P. Puccetti, and F. Bistoni. 1993. $CD4^+$ subset expression in murine candidiasis. *J. Immunol. 150:925.*
38. Heinzel, F. P., M. D. Sadick, B. J. Holaday, R. L. Coffman, and R. M. Locksley. 1989. Reciprocal expression of IFN-γ or interleukin 4 during the resolution or progression of murine leishmaniasis: evidence for expansion of distinct helper T cell subsets. *J. Exp. Med. 169:59.*
39. James, S. L., C. Salzman, and E. J. Pearce. 1988. Induction of protective immunity against *Schistosma mansoni* by a non-living vaccine. VI. Antigen recognition by non-responder mouse strains. *Parasite Immunol. 10:71.*